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TITLE: Identifying Early Events in BRCA2-Related Breast Cancer Using Isogenic Cell Lines

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13. ABSTRACT (Maximum 200 Words)

Three paired primary cell lines derived from BRCA- mutation carrying women have been established. These lines are B28T, B28N (*BRCA2* mutation), B22T and B22N and B23T and B23N (*BRCA1* mutation) where T and N indicates that the cells are originated from tumor and normal tissue respectively. After mincing and separation, the cells were transformed with E6/E7. The cells were growth in Basal Epithelial media (BEM) supplemented with Insulin, Epidermal Growth Factor and glucocorticoids for the first 2 passages and subsequently with RPMI medium supplemented with 15% fetal bovine serum (FBS). A few B28T cells expressed AE1/AE3 (MCF-7 positive control). This results indicates that only 5% of B28T cells express epithelial markers and suggest that the other cells are mesenchymal (Vimentin +) or myoepithelial cells (Actin +). CK 8/18 and CK 5/6 were used to look for basal differentiation. The only preparation of cells that was positive for CK5/6 was B28T. We concentrate our efforts on the B28T cell line to amplify the subpopulation of cytokeratin positive cells. This line, when fully characterized, will be of considerable value to the research community. Now that we have established this line, further characterization will now follow. We did not complete our goals for this award, but will continue to work on this line for the coming years.

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Identifying early events in *BRCA2*-related breast cancer using isogenic cell lines

Introduction

With this award, we hoped to be able to characterize a unique cell line, B28T, and its companion cell line, B28N. These two cell lines are derived from cells from the same woman, who carries a *BRCA2* mutation. These cell lines are unique because no breast cell lines derived from *BRCA2* carriers are currently available. There is one *BRCA2* mutant cell line available, but this is from a patient with pancreas cancer. Detailed characterization of these two lines could provide new insights into carcinogenesis in *BRCA2* gene carriers.

Body

Based on the statement of work

Task 1: Detailed molecular characterization of paired BB28N/BB28T breast cell lines from germ-line *BRCA2* mutation carrier (months 1-12)

a) Cellular localization of BRCA2. Commercially available monoclonal antibodies to BRCA2 (amino-terminus) will be used to confirm the expected cytoplasmic location of truncated BRCA2 (months 1-2)

Status: partly completed- in progress (repeat experiments required). See appendix- Figure 1 shows that both normal and tumor lines express E6/E7. Figure 2 shows the Western blots- these need to be repeated.

b) Loss of heterozygosity studies. Single nucleotide polymorphisms (SNPs) will be identified using web-based resources and oligonucleotide primers which flank the SNPs will be synthesized. Initial focus will be on chromosomes 13 and 17 (months 1-4, concurrent with a).

Status: in progress

c) Mutation analysis. Existing oligonucleotide primers that flank the exons (genomic sequencing) or fragments of the cDNA (cDNA sequencing) for the relevant genes (*BRCA2*, *TP53*, *PTEN*, *and CDKN2A*) will be synthesized. Automatic sequencing will be performed (months 4-8).

Status: in progress

d) Immunocytochemistry. Commercially available antibodies raised against p53, ER,p27Kip1, cyclin E and HER2 will be purchased and used to immunostain the two cell lines (months 1-6, concurrent with a-c).

Status: partly completed, in progress (see appendix Figures 3-6, Table 1)

e) Karotypic analysis. Cells will be prepared for karotype analysis by spectral karyotyping, G banding and FISH as indicated (months 6-8)

Status: to be performed.

Task 2: Analysis of differences in DNA repair capacity in the paired cell lines (BB28N/BB28T) (months 6-12, to run concurrently with several parts of Task 1).

Status: delayed- to be performed.

a) Formation of Rad51 foci. Cells will be prepared for irradiation, and Rad51 foci will be quantified and co-localization with anti-RPA and anti-BRCA1 will be performed (months 6-7)

b) DNA damage sensitivity. Cells will be exposed to γ-radiation, UV light, cisplatinum and mitomycin C. Cell death will be measured in the two cell lines (months 7-12).

Status: delayed- to be performed.

Delays occurred for Task 2 because Task 1 has taken us longer to complete than expected.

Key Research Accomplishments

- Establishment and preliminary characterization of a mammary epithelial cancer cell line from a *BRCA2* gene carrier

Reportable outcomes

1. Cell line development

Two *BRCA1*-related breast cancer isogenic cell lines are being developed: B22T and B22N and B23T and B23N (BRAC1 mutant cell lines) where T and N indicates that the cells are originated from tumor and normal tissue respectively. BRCA1 lines have also been developed (appendix, Figure 7).

2. Funding applied for, or obtained

a) Funding applied for: "Response To Chemotherapeutic Agents Of A Human Brca2 Breast Cancer Cell Line: The Role of Trophic Factors" Congressionally Directed Research Program (CDRMP) Idea-Grant-Breast Cancer Research Program. Dr R Aloyz, Principal Investigator, Dr WD Foulkes Co-Investigator.

b) Funding applied for: Burroughs Wellcome Fund: Molecular approaches to improving

the management of women with *BRCA1* mutations (PI: WD Foulkes).

c) Funding obtained: Hereditary Breast and Ovarian Cancer Scholar 2004 awarded to Mr. Kyle White. Summer Project: "Immunochemical characterization of an epithelial breast BRCA2 cell line". Supervisor Dr Aloyz R and Dr Panasci L. Lady Davis Institute for Medical Research.

Training supported by this award

a) Summer student (see b. above). Mr. White (a second year Bachelor student at McGill University in Biochemistry) leaned the principles of tissue culture and immunological techniques. He characterizes the cytokeratin expression in B28T cells by immunofluorecence. Furthermore he assessed the expression of BRAC2 in B28T cells by western blot. (See appendix figures)

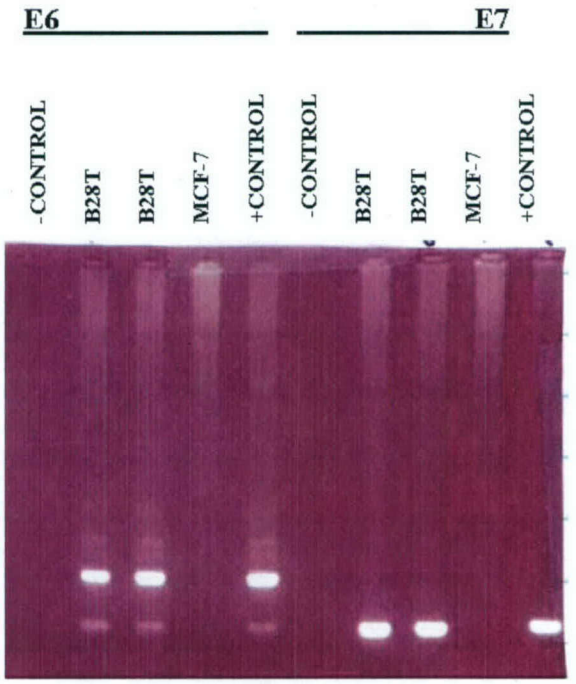
Conclusion

We have established a breast cancer cell line from a *BRCA2* gene mutation carrier. This unique cell line will form the basis of our investigations into early events in *BRCA2*related breast cancer.

References

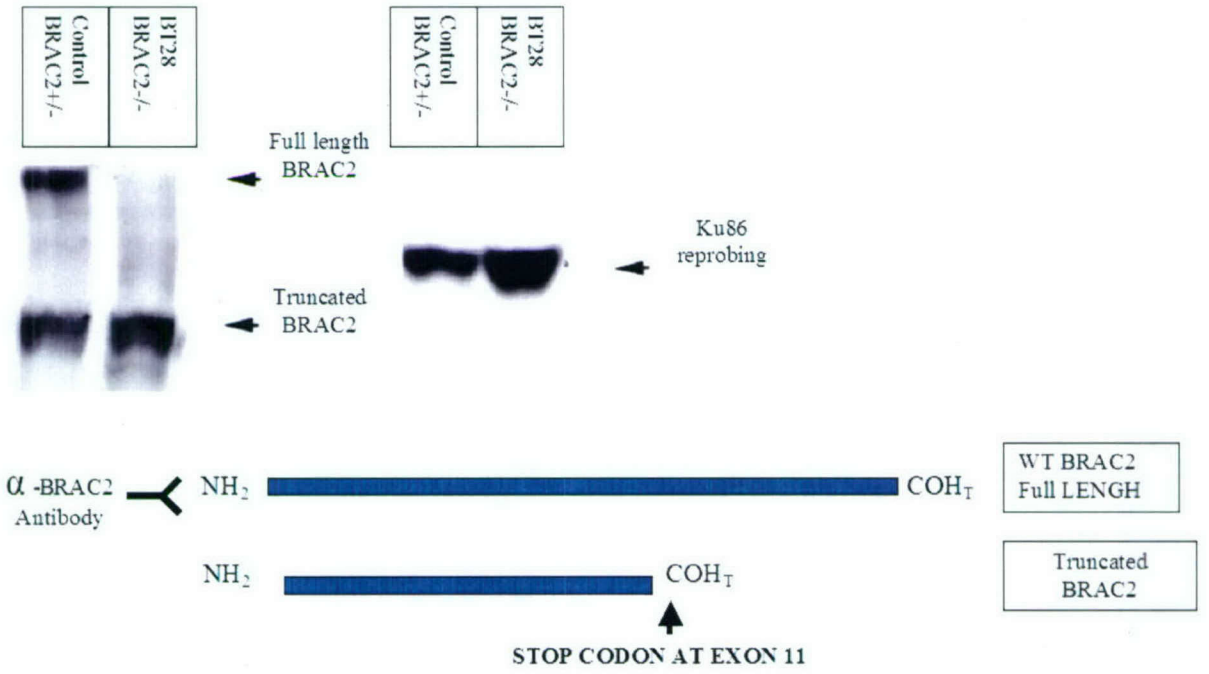
1. Kyriazis AP, Kyriazis AA, Scarpelli DG, Fogh J, Rao MS, and Lepera R: Human pancreatic adenocarcinoma line Capan-1 in tissue culture and the nude mouse: morphologic, biologic, and biochemical characteristics. Am J Pathol 1982, 106: 250-26

FIGURE 1 B28N and B28T cells express E6/E7



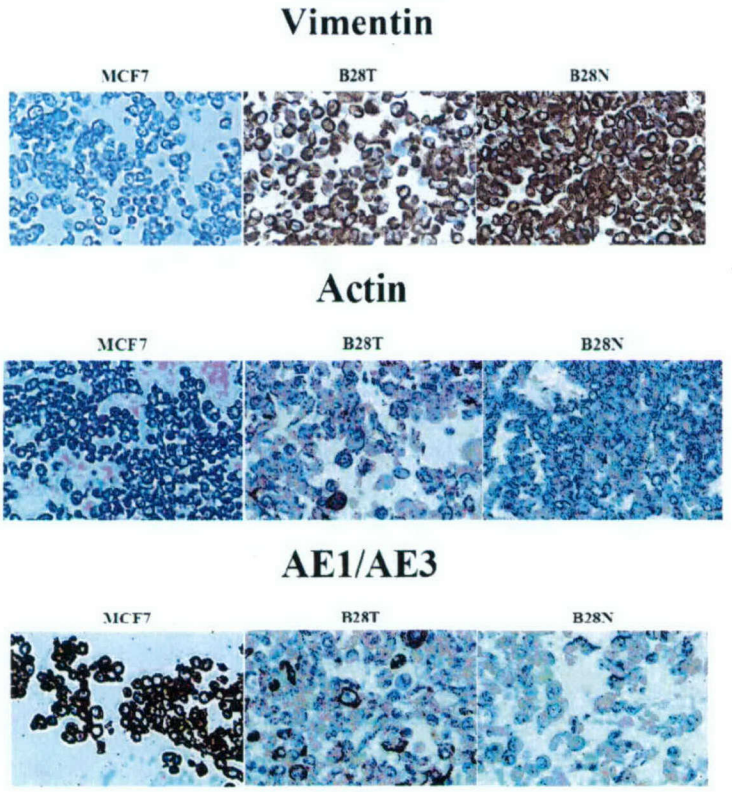
A portion of the primary tumor and normal tissue (invasive ductal breast carcinoma) were minced and scraped to release tumor cells and placed in RPMI medium with 15% serum and 5% CO_2 and E6/E7 transformed. Specific primers were designed to assess the expression of E6/E7 in B28T and B28N cells by RT-PCR. MCF-7 cells were used as negative control and Hela cells as positive control.

FIGURE 2 BRCA2 profile expression of B28T and B28N cells



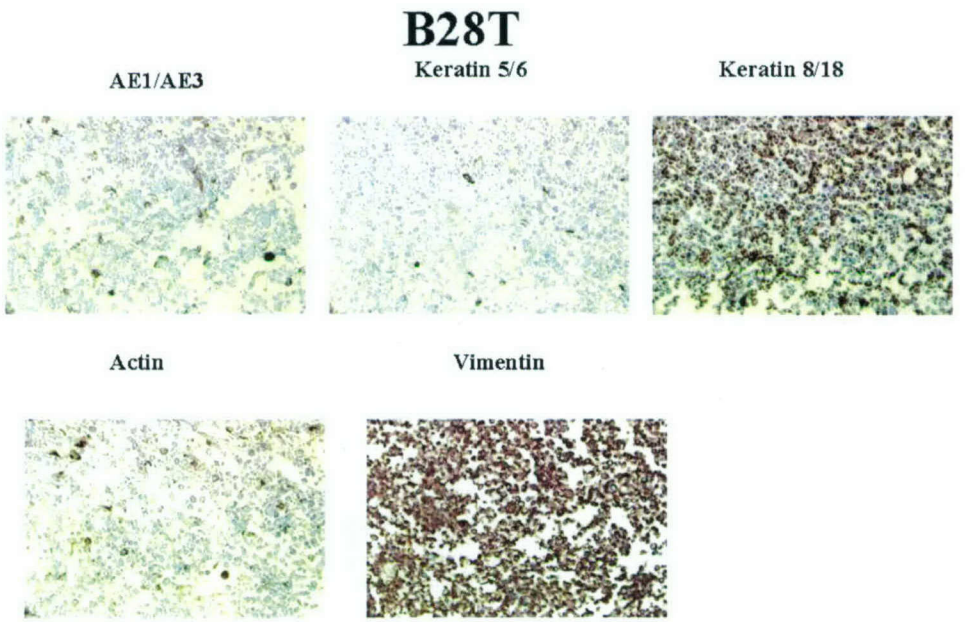
B28T cells (BRCA 2 -/-) and B28N cell BRCA2 +/-) pellets were lysed in the presence of 20mM Tris-HCl pH 8, 135 mM NaCl, 1% NP-40, 10% Glycerol, 0.01% SDS containing proteases inhibitors (Roche proteases inhibitor cocktail) and NaVanadate. Proteins extracts (100 µg) were separated by a 410% SDS-PAGE gradient gel, transferred to a nitrocellulose membrane and probed with a BRCA2 specific antibody recognizing BRCA2- NH_2 Terminus (Novus Biological). The membranes were re probed with Ku86 antibody to assess equal loading.

FIGURE 3 Immunocytochemical Characterization of B28T and B28N cells at Passage 10



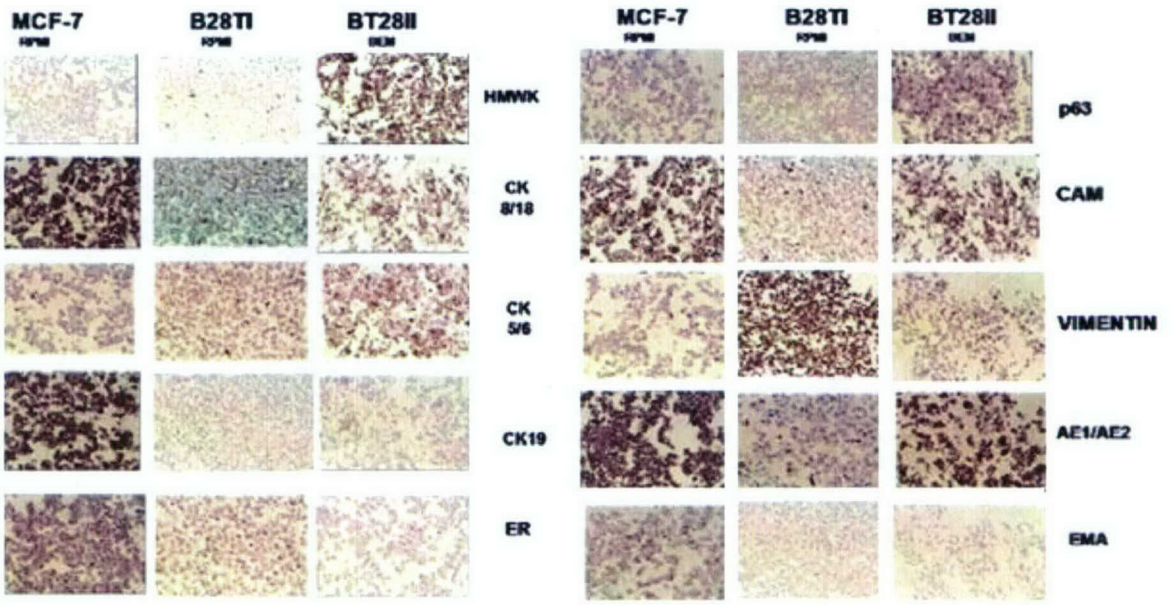
The cells were subcultured by trypsinization every 2 weeks. At passage 10, the cells were collected mechanically, imbibed in a paraffin block and analyzed immunocytochemically for the expression of Vimentin (Dako, clone 34B4; mesenchymal marker), Actin (Dako, clone HHF35; myoepithelial marker) and AE1/AE3 (Dako clone AE1/AE3; a luminal marker). MCF-7 cells (a breast carcinoma cell line was used as a positive control for AE1/AE3). The preparations were developed using streptavidin/biotin (Vectastin ABC kit). Brown color indicates the presence of the corresponding antigen. B28T cells express epithelial and mesenchymal markers while B28N cell express only mesenchymal markers, suggesting that B28N cells are fibroblasts.

FIGURE 4 B28T cells express Basal epithelial markers



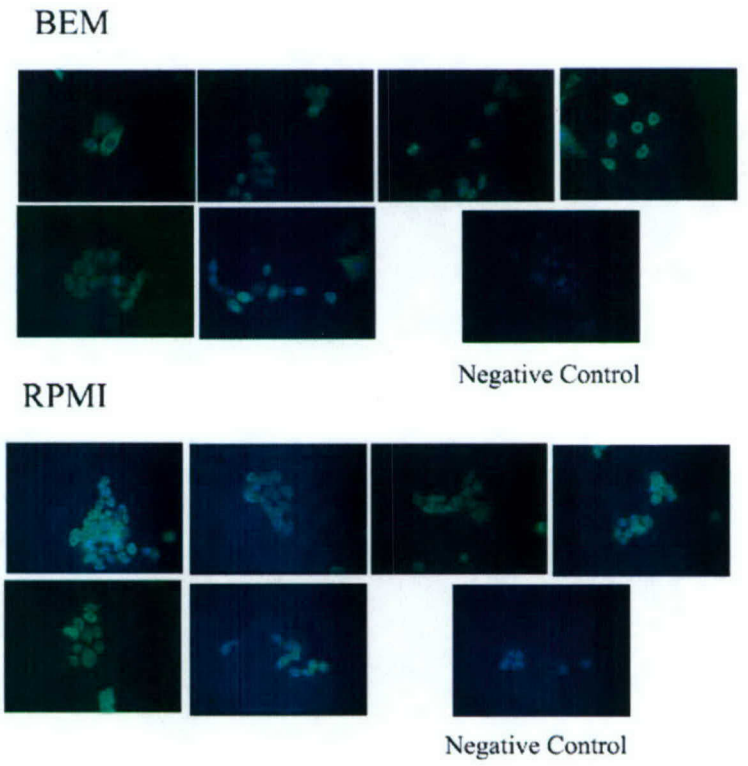
The results shown in Figure 3, indicates that 5% of B28T cells express epithelial markers and suggest that the other cells are mesenchymal (Vimentin +) or myoepithelial cells (Actin +). To asses the presence of basal epithelial cells we assess the expression in the same preparation of cells of CK 8/18 (Neomarkers clone K8.8+DC10) and CK 5/6 (Neomarkers Ab2 clones D5/16B4).

FIGURE 5 Differential markers expressed by B28T cells in RPMI or BEM media (20 passages)



After passage 10, the cells were passage every week. B28T cells were grown in RPMI/15% FBS or BEM media (basal epithelial media). At passage 20, the cells were scraped and imbibed in paraffin block and analyzed immunocytochemically for the expression of cytokeratins, ER, PR, p63, and β-actin. We found that all the cells expressed HMW keratins (1, 5, 10 and 14) and p63. Moreover, the cells were negative for the β-actin, expressed in myoepithelial cells and for CK19 and EMA expressed in luminal epithelial cells. Interestingly, some cells, expressed cytokeratin 8/18. Even though most breast cancer cells have either luminal (CK8/18) or basal (HMWCK) phenotype, some tumors express both markers. ER and PR staining was not detected. Interestingly, while BT28 cells grow in RPMI media supplemented with 15% serum, they are not insensitive to external cues since they grow faster in defined media (Basal Epithelia Media, BEM) supplemented with EGF, insulin and glucocorticoids. Moreover, there were more cells staining for cytokeratins and p63 in cells grown in supplemented BEM in comparison to RPMI media supplemented with 15% serum.

FIGURE 6 At Passage 29 B28T cells express epithelia markers in both, RPMI or BEM media



B28T cells were plated in coverslips and maintained in RPMI or BEM media. Subconfluent cultures were fixed and probed with anti- pan Keratin ab-2 (Neomarkers): 4, 5, 6, 8, 10, 12, 14, 15, and 16 18 and 19. The DNA was counterstained with DAPI.

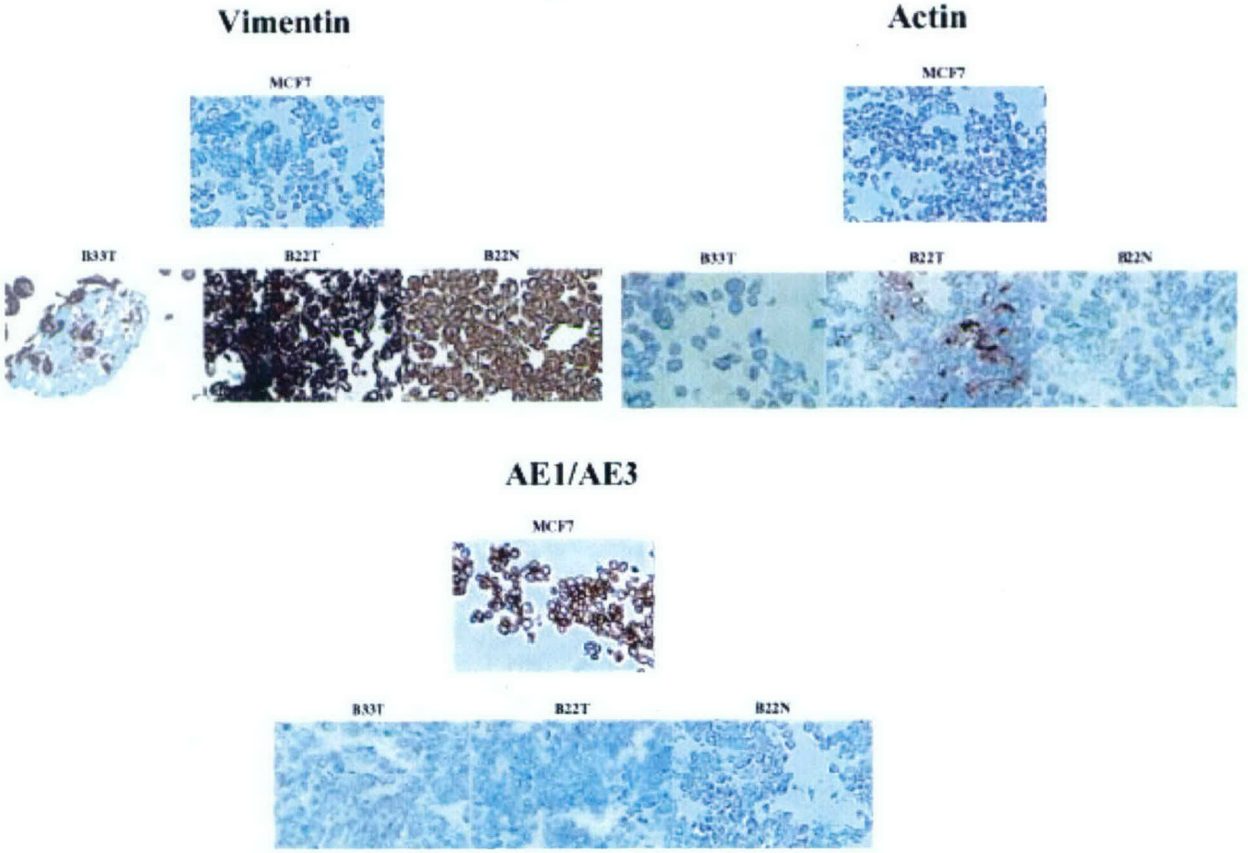
TABLE 1 Epithelial markers expressed by B28T cells at passage 28

MARKER	Passage 29th
VIMENTIN	-
ACTIN	-
P63	+
HMWK	+
ck 5/6	+
CAM(ck 8/18)	+
ck 7	+
ER	+/-
E-Catherin	+/-
ck 19	-
ck 20	-
AE1/AE3	+/-
EMA	-

th

After passage 29the cells were collected mechanically, imbibed in a paraffin block and analyzed immunocytochemically, the expression of the marker indicated were quantified as follows: No labeling -; > 70% labeling +; < 70% labeling +/-.

FIGURE 7 Two *BRCA1*-related breast cancer isogenic cell lines (B22 and B23) express mesenchymal markers



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